

Genetic Diversity in the Protective Antigen Gene of *Bacillus anthracis*

LANCE B. PRICE,¹ MARTIN HUGH-JONES,² PAUL J. JACKSON,³ AND PAUL KEIM^{1*}

¹Department of Biological Science, Northern Arizona University, Flagstaff, Arizona 86011-5640; ²Department of Epidemiology and Community Health, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana 70803; and ³Environmental Molecular Biology Group, Los Alamos National Laboratory, Los Alamos, New Mexico 87545

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Bacillus anthracis is a gram-positive spore-forming bacterium that causes the disease anthrax. The anthrax toxin contains three components, including the protective antigen (PA), which binds to eucaryotic cell surface receptors and mediates the transport of toxins into the cell. In this study, the entire 2,294-nucleotide protective antigen gene (*pag*) was sequenced from 26 of the most diverse *B. anthracis* strains to identify potential variation in the toxin and to further our understanding of *B. anthracis* evolution. Five point mutations, three synonymous and two missense, were identified. These differences correspond to six different haplotypes, which translate into three different amino acid sequences. The two amino acid changes were shown to be located in an area near a highly antigenic region critical to lethal factor binding. Nested primers were used to amplify and sequence this same region of *pag* from necropsy samples taken from victims of the 1979 Sverdlovsk incident. This investigation uncovered five different alleles among the strains present in the tissues, including two not seen in the 26-sample survey. One of these two alleles included a novel missense mutation, again located just adjacent to the highly antigenic region. Phylogenetic (cladistic) analysis of the *pag* corresponded with previous strain grouping based on chromosomal variation, suggesting that plasmid evolution in *B. anthracis* has occurred with little or no horizontal transfer between the different strains.

Bacillus anthracis is the causative organism of the potentially fatal disease anthrax. Virulent forms of *B. anthracis* carry two large plasmids, pX01 (ca. 174 kb) and pX02 (ca. 95 kb). Virulence factors include toxin and capsule production, encoded on pX01 and pX02, respectively. The anthrax toxin is composed of three proteinaceous subunits: (i) lethal factor (LF), the toxin component thought to kill host cells by disrupting the mitogen-activated protein kinase pathway (2); (ii) edema factor (EF), an adenylate cyclase that causes skin edema in the infected host (6); and (iii) protective antigen (PA), which binds to eucaryotic cell surface proteins, forms homoheptamers, and then binds to and internalizes EF and LF.

The structure and function of PA have been well described. The entire PA gene (*pag*) sequence has been published and is available in GenBank (accession no. M225S9) (12). The three-dimensional structure has also been solved and is available in the NCBI Entrez 3D database (MMDB no. 6980) (10). Finally, antibody-binding experiments have been used to define regions of the PA protein critical to cell surface attachment as well as LF binding (8). Missing from the literature until now was a population study of *pag* from diverse strains of *B. anthracis* to define the natural variation in this important gene.

In past studies, plasmid-specific genetic variation in *B. anthracis* has been largely ignored. A recent population study, based on chromosomal markers, demonstrated that *B. anthracis* is one of the most monomorphic bacterial species known (5). This chromosomal amplified fragment length polymorphism study examined ca. 6.3% of the *B. anthracis* genome for length variations and ca. 0.36% for point mutations. However, due to ambiguities arising from the absence of one or both of

the plasmids, plasmid data were omitted from the final results. Studies of pX01 diversity and especially of *pag* are essential to understanding evolution of pathogenesis in *B. anthracis*. Likewise, comparative studies of plasmid-based versus chromosomal variation can provide insight into the frequency of horizontal plasmid transfer in natural *B. anthracis* populations.

In this study we have sequenced the entire *pag* gene from 26 of the most diverse strains of *B. anthracis* (5). These sequences were aligned and analyzed for point mutations then studied phylogenetically to determine if the *pag* data are consistent with chromosomal diversity groups. Additionally, we sequenced a 307-bp variable region of *pag* from 10 Sverdlovsk anthrax victim necropsy samples (4) in order to identify novel *pag* sequences.

MATERIALS AND METHODS

B. anthracis DNA. Culture conditions, DNA isolation methods, and diversity groups are described in reference 5. Necropsy tissue DNA was isolated as described by Jackson et al. (4).

PCR amplification of DNA. Table 1 contains the sequences for all primers used for this project. These were designed from the published *pag* sequence (GenBank accession no. M225S9) and synthesized by Gibco/BRL, Bethesda, Md. All primer positions cited throughout this report are based on this GenBank sequence. Two DNA fragments, together totaling 2,531 bp of sequence, were initially amplified to provide a *pag* sequencing template from the 26 *B. anthracis* strains. PA-1F and PA-1R were used to amplify a 1,191-bp fragment containing the 3' portion of PA. This included 131 bp of upstream flanking sequence. PA-2F and PA-2R were used to amplify a 1,449-bp fragment containing the 3' portion of PA. This included 106 bp of downstream flanking sequence. The two fragments contained 109 bp of overlapping sequence near the middle of the gene. Fifty-microliter PCR mixtures contained 1× PCR buffer (20 mM Tris [pH 8.8], 50 mM KCl, Gibco/BRL), 0.10 mM deoxynucleoside triphosphates, 4 mM MgCl₂, 0.2 ng of template DNA per μ l, 0.04 U of *Taq* DNA polymerase (Gibco/BRL) per μ l, and 0.4 μ M forward and reverse primers, adjusted to 50 μ M with filtered (0.2- μ m pore-size filter) 17.8 mM E-pure water. Reactions were heated to 94°C for 5 min and then subjected to 35 cycles, each consisting of 30 s at 94°C, 30 s at 62°C, and 1.5 min at 72°C. This was followed by heating to 72°C for 5 min to complete primer extension. PCR products were purified through

Primer

PA-1F
PA-1R
PA-2F
PA-2R
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PA-4
PA-5F
PA-5R
PA-5F_{nest}
PA-5R_{nest}

* Amp, used for

† NA, not applic

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* Corresponding author. Mailing address: Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ 86011-5640. Phone: (505) 523-1078. Fax: (520) 523-7500. E-mail: Paul.Keim@nau.edu.

TABLE 1. Primers used in this study

Primer	Type ^a	Sequence (5'→3')	Position ^b	Amplicon size (bp)
PA-1F	Amp/Seq	ATA TTT ATA AAA GTT CTG TTT AAA AAG CC	5'-1673	1,191
PA-1R	Amp/Seq	TAA ATC CTG CAG ATA CAC TCC CAC	3'-2840	1,191
PA-2F	Amp/Seq	ATA AGT AAA AAT ACT TCT ACA AGT AGG ACA C	5'-2755	1,449
PA-2R	Amp/Seq	GAT TTA GAT TAC TGT TTA AAA CAT ACT CTC C	3'-4173	1,449
PA-3	Seq	TCA TGT AAC AAT GTG GGT AGA TGA C	5'-2145	NA ^c
PA-4	Seq	CTC TAT GAG CCT CCT TAA CTA CTG AC	3'-3717	NA
PA-5F	Amp	ATC CTA GTG ATC CAT TAG AAA CGA C	5'-3416	330
PA-5R	Amp	CTT CTC TAT GAG CCT CCT TAA CTA CTG	3'-3719	330
PA-5F _{mut}	Amp/Seq	AGT GAT CCA TTA GAA ACG AC	5'-3421	307
PA-5R _{mut}	Amp/Seq	TAA CTA CTG ACT CAT CCG C	3'-3709	307

^a Amp, used for amplification; Seq, used for sequencing; Amp/Seq, used for both amplification and sequencing.^b All correspond to GenBank accession no. M22589 nucleotide positions.^c NA, not applicable.

Qiaquick purification minicolumns (Qiagen Inc., Valencia, Calif.) and then quantified on ethidium bromide-stained 1.25% agarose-Tri-acetate-EDTA gels. These purified fragments were then used in subsequent sequencing reactions. PCR amplification of necropsy sample DNA was performed as described by Jackson et al. (4), using primers PA-5F, PA-5R, PA-5F_{mut}, and PA-5R_{mut} (Table 1).

DNA sequencing. PCR products were sequenced on an ABI model 377 fluorescence sequencer using a PRISM Ready Reaction BigDye terminator cycle sequencing kit (both from Perkin-Elmer/Applied Biosystems Inc., Foster City, Calif.). Sequences were aligned and analyzed with Sequence Navigator software (Perkin-Elmer/Applied Biosystems).

Claustic analysis. Cladistic analysis was performed on the *pag* sequences by using maximum parsimony with PAUP 3.1.1 software (developed by David L. Swofford, Illinois Natural History Survey) and manual examinations of sequence polymorphisms.

Three-dimensional analysis. The PA structure has been solved and is available on the NCBI Entrez 3D database (MMDDB no. 6980) (10). Amino acid residues shown to vary among strains were identified on the three dimensional structure, and then physical distances from the putative LF binding region of PA domains

3 and 4 were estimated by using MAGE 4.5 software (developed by David Richardson, Biochemistry Department, Duke University, Durham, N.C.).

RESULTS

Sequence alignment of the entire PA gene from 26 strains representative of the five *B. anthracis* diversity groups (5) (Table 2) revealed five point mutations, three synonymous and two missense, shown in Table 3. All five mutations are transitions. Two of the synonymous mutations occur only once. However, the other differences are present with frequencies ranging from 3/26 to 20/26. The two missense mutations are located adjacent to a highly antigenic region crossing the junction between PA domains 3 and 4 shown to be critical to LF binding (Fig. 1) (8, 10). The different mutational combinations observed in this

TABLE 2. *B. anthracis* strains used in this study

Strain	Geographic origin or description	Diversity group ^a	PA genotype ^b	PA phenotype ^c
BA0052	Jamaica	Sterne-Ames	I	FPA
BA1087	Scotland	Sterne-Ames	I	FPA
3611	Indonesia	Sterne-Ames	I	FPA
BA1031	South Africa	Sterne-Ames	I	FPA
BA1043	South Africa	Sterne-Ames	I	FPA
28	Ohio	Sterne-Ames	II	FPA
MOZ-3	Mozambique	Southern Africa	III	FPA
BA1035	South Africa	Southern Africa	III	FPA
33	South Africa	Southern Africa	IV	FPA
A24	Slovakia	Southern Africa	V	FPV
K20	South Africa (Kruger)	Kruger	V	FPV
26/05/94	Zambia	Kruger	V	FPV
BA1033	South Africa	WNA	V	FPV
BA1017	Haiti	WNA	V	FPV
BA1015	Maryland	WNA	V	FPV
93-194C	Canada	WNA	V	FPV
93-195C-8	Canada	WNA	V	FPV
BA1040	Colorado	WNA	V	FPV
BA1007	Iowa	WNA	V	FPV
26	Turkey	WNA	V	FPV
Pak-2	Pakistan	WNA	V	FPV
STI-1	Russian vaccine strain	WNA	V	FPV
F-1	South Korea	Vollum	V	FPV
BA1024	Ireland	Vollum	VI	FSV
ASC-3	United Kingdom	Vollum	VI	FSV
BA1009	Pakistan	Vollum	VI	FSV

^a Diversity designations are consistent with those described by Keim et al. (5).^b Described in Table 4.^c Designated by the single-letter designations of the three amino acids shown to vary in this study.

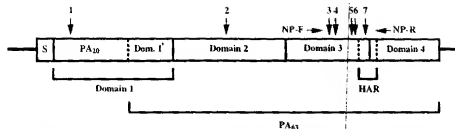


FIG. 1. Model of *pag* from *B. anthracis*. S, region of gene that codes for cleaved signal region; NP-F and NP-R, forward and reverse nested primers used to amplify variable regions from the Sverdlovsk tissue samples; black vertical arrows, missense mutations; grey vertical arrows, synonymous mutations; HAR, highly antigenic region important to LF binding (8, 10). Dom., domain.

study give rise to six PA genotypes and three PA phenotypes (Table 4).

Cladistic analysis of the 26 *pag* sequences was performed by the maximum parsimony method to produce a gene tree (Fig. 2A). The 26 strains grouped into four clades of 3, 3, 6, and 14 individuals. These groups were defined by three synapomorphic (informative) differences. In addition, we identified two apomorphic (uninformative) nucleotide differences (mutations 2 and 6) that separated two strains (28 and 33) from others in their clades. These mutations are identified on the respective branches but were not used to isolate these strains from their groups. The clades and topology identified by this tree were mostly congruent with those generated from chromosomal markers (Fig. 2B) (5). The only aberrations are the following. (i) Chromosomal data from strain A24 indicate that it is of the Southern Africa lineage (S), but the *pag* data place this strain with the Western North America (WNA) diversity group (one mutational step away); (ii) chromosomal data from strain F-1 indicate that it is of the Vollum lineage, but the *pag* data place this strain with the WNA diversity group (again, one mutational step away); and (iii) chromosomal markers indicate that the Kruger samples, although very similar, are genetically distinct from the WNA lineage. However, the *pag* gene tree did not resolve these two distinct groups. It should be noted that chromosomal markers indicate that Vollum and WNA are sister groups and, likewise, that Kruger and WNA are closely related. Only with strain A24 do the *pag* data suggest that strains from two distantly related groups (based on chromosomal markers) are closely related.

To determine the *pag* genotypes and phenotypes of the strain(s) involved in the Sverdlovsk incident, nested PCR primers (Table 1) were designed to amplify and sequence a 307-bp region of *pag*. This region spans the junction between PA domains 3 and 4 where much of the variation was observed. This analysis uncovered two additional transition mutations (3 and 7 in Table 3). One was synonymous, while the other was a

novel missense mutation resulting in a phenylalanine \rightarrow leucine change. These changes resulted in two additional genotypes and one new phenotype (Table 4). The amino acid change was, again, immediately adjacent to the highly antigenic region of PA domains 3 and 4 (Fig. 1). Repetitive sequencing of these tissues uncovered multiple PA genotypes within some of the individual necropsy samples. Together, five different PA genotypes were observed in the Sverdlovsk samples, with some samples showing evidence of infection by multiple strains (Table 5). This finding is consistent with the results of Jackson et al. (4).

Figure 3 is an unrooted phylogenetic tree demonstrating the five mutational steps leading to the six PA genotypes and three PA phenotypes identified in this study. Additionally, the putative positions of the Sverdlovsk samples are shown. However, because the Sverdlovsk identifications were based on just the 307-bp region around the antigenic portion of PA domains 3 and 4, these placements are only tentative.

Three-dimensional analysis of all the amino acid changes observed in this study (mutations 3, 4, and 5 in Table 3) indicated that these changes are not only close sequentially but also very close in three-dimensional space to the antigenic region important for LF binding. Mutation 3 (Phe to Leu), is ca. 11.2 Å, mutation 4 (Pro to Ser) is ca. 20.3 Å, and mutation 5 (Ala to Val) is ca. 19.0 Å from the central portion of this

PA genotype

I
II
III
IV
V
VI
VII_{old}
VIII_{old}

* NA, not applicable
† Designated by
‡ Described in T

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The proteolysis associated with its encoding *pag* *anthracis* virus sequenced in (12). In this sequence (2,294 only five point genetic mono Among of the missense (1 ratio of miss: 1:5; here we see missense mutation critical to LF A, demonstr that Ile-581 LF binding t the miss

TABLE 5.

Sample	Genotype
15.15	
31.40.5	
32.30.3	
33.35.4	
33.35.6	
34.1	
35.031	
36.21	
37.20.5	
38.4	

TABLE 3. Mutations identified in this study

Mutation	Nucleotide position*	Base change	Frequency	Amino acid change
1	1998	C \rightarrow T	20/26	Synonymous
2	2883	G \rightarrow A	1/26	Synonymous
3	3481	T \rightarrow C	NA ^b	Phe \rightarrow L
4	3496	C \rightarrow T	3/26	Phe \rightarrow S
5	3602	C \rightarrow T	17/26	Asp \rightarrow V
6	3606	T \rightarrow C	1/26	Synonymous
7	3672	A \rightarrow G	NA	Synonymous

* Nucleotide positions are based on the 4,235-bp pX01 sequence from Sterne strain, accession no. M22589, containing *pag* in its entirety.

^b NA, not applicable; mutations were observed only in the Sverdlovsk samples.

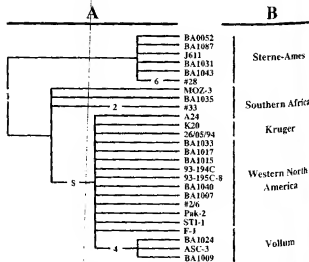


FIG. 2. Cladistic analysis of the 26 diverse strains. (A) Unrooted, maximum parsimony gene tree based on *pag* data described in this study. (B) Strain diversity groups based on chromosomal AFLP data described by Keim et al. (5). Branch mutations are numbered as described for Table 3.

TABLE 4. PA genotypes and phenotypes identified in this study^a

PA genotype	PA phenotype ^b	Genotypic frequency	Mutation ^c						
			1	2	3	4	5	6	7
I	FPA	5/26	C	G	T(F)	C(P)	C(A)	T	A
II	FPA	1/26	C	G	T(F)	C(P)	C(A)	C	A
III	FPA	2/26	T	G	T(F)	C(P)	C(A)	T	A
IV	FPA	1/26	T	A	T(F)	C(P)	C(A)	T	A
V	FPV	14/26	T	G	T(F)	C(P)	T(V)	T	A
VI	FSV	3/26	T	G	T(F)	T(S)	T(V)	T	A
VII _{iso}	LPA	NA	—	—	C(L)	C(P)	C(A)	T	A
VIII _{iso}	LPA	NA	—	—	T(F)	C(P)	C(A)	T	G

^a NA, not applicable (mutation was seen only in the Sverdlovsk samples). —, the region was not analyzed for the Sverdlovsk sample.^b Designated by the single-letter designation of the three amino acids shown to vary.^c Described in Table 3.

region. These spatial distances were estimated solely on peptide backbone-to-peptide backbone relationships. However, when the three-dimensional spaces occupied by the side chains of the amino acids were considered, changes were found to affect residues as close as 6.9 Å from the central amino acids of this critical antigenic region.

DISCUSSION

The protective antigen protein is central to the virulence associated with anthrax toxin. Elucidation of PA variation and its encoding gene could lead to a better understanding of *B. anthracis* virulence and evolution. Until now, *pag* had been sequenced in its entirety only from a single *B. anthracis* strain (12). In this study, a detailed analysis of the entire *pag* sequence (2,294 bp) from 26 diverse *B. anthracis* strains revealed only five point mutations, corroborating the high degree of genetic monomorphism found by Keim et al. (5).

Among these mutations, there is a disproportionate number of missense (two) to synonymous (three) changes. A common ratio of missense to synonymous mutations is approximately 1:5; here we see a ratio more than threefold greater (7). These missense mutations are located near a highly antigenic region, critical to LF binding. In monoclonal antibody studies, Little et al. demonstrated that by blocking an epitope between amino acids Ile-581 and Asn-601 (Fig. 1), they could effectively block LF binding to PA (8). Three-dimensional analysis indicated that the missense mutations identified in our study are very

close in three-dimensional space to this antigenic region. While none of the three missense mutations were dramatic, such as a change from an extremely hydrophobic to a hydrophilic amino acid, the proline-to-serine change has the potential to make important three-dimensional alterations, since proline isomerization is known to play a critical role in protein folding. Because of their close proximity, these amino acid changes have the potential to effect LF binding, either directly or indirectly, within an infected host. The grouping of these missense mutations near this antigenic region and the disproportionate number of missense to synonymous mutations suggests adaptive variation. One of the two new mutations identified in the Sverdlovsk victims' tissues was found to be a novel missense mutation located, sequentially and three dimensionally, near the highly antigenic region of the junction between PA domains 3 and 4. When these mutations are included with those identified in the 26-sample survey, the ratio of missense to synonymous mutation is increased to 3.8:5.

The amplification and sequencing of the 307-bp *pag* fragment from the Sverdlovsk tissue samples suggested that at least five different strains of *B. anthracis* were present in the samples and that some of the individual victims had been infected with multiple strains. These data corroborate earlier work with the *verA* locus that suggested that multiple strains of anthrax had been released during the 1979 incident (1, 4). Besides the Russian vaccine strain STI-1, included in this study, these tissue samples are a rare glimpse at the different strains of *B. anthracis* that are thought to be endemic in the vast region of the former Soviet Union. The fact that two previously unobserved mutations were found in the Sverdlovsk samples stresses the importance of collecting and analyzing *B. anthracis*

TABLE 5. Tissue samples from Sverdlovsk victims analyzed in this study^a

Sample	Tissue	PA genotype(s)	PA phenotype(s)
7 RA93.15.15	Spleen	V	FPV
40 RA93.40.5	Spleen	VI, VII _{iso}	FSV, LPA
27 RA93.30.3	Spleen	V	FPV
37 RA93.35.4	Vaccination site	I ^b , V	FPA, FPV
37 RA93.35.6	Lung	VIII _{iso}	FPA
3 RA93.1.1	Meninges	VIII _{iso}	FPA
25 RA93.031	Meninges	V	FPV
1 RA93.42.1	Meninges	V	FPV
33 RA93.20.5	Meninges	V	FPV
21 RA93.38.4	Lymph node	V	FPV

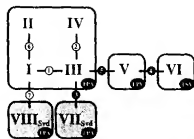
^a Determination of PA genotypes and phenotypes was based solely on the 307 bp region connecting PA domains 3 and 4. Multiple strains were identified in some tissues.^b Due to the limited region analyzed, this strain may be type I, II, or IV but was grouped with I for simplicity.

FIG. 3. Unrooted phylogenetic tree of PA genotypes. Open boxes show the three PA phenotypes identified; shaded boxes show the possible positions of the Sverdlovsk genotypes, VII_{iso} and VII_{iso}. Synonymous mutations are shown in open circles, and missense mutations are shown in closed circles. Each mutation is described in Table 3 and the phenotypes are described in Table 4.

strains from areas where anthrax is endemic but largely uncharacterized by molecular genetic analysis.

Independent cladistic analysis of pX01 by using the *pag* sequence has enabled us to estimate the likelihood of horizontal transfer of this plasmid between different *B. anthracis* strains in natural populations. Although horizontal transfer in *Bacillus* spp. is possible under laboratory conditions, the similarity of the cladistic grouping from the *pag* data to that of the chromosomal markers suggests that the differences in *pag* arose from evolution within particular strain lineages and were not a result of horizontal pX01 transfer. The single possible exception is associated with the A24 sample, which chromosomally is related to the Southern Africa strains, while the *pag* data for this strain are consistent with Kruger-WNA. This is either a result of convergent evolution or evidence of horizontal pX01 transfer. Further, it should be noted that the data presented in this report do not rule out the potential for horizontal transfer of plasmid pX01 between closely related strains within an infected host.

The unrooted phylogenetic tree (Fig. 3) is a useful tool for demonstrating the relationships between the different PA genotypes. However, it is not meant to infer an evolution toward a particular form of PA. Although distant homologues from other gram-positive bacteria are cited (3, 9, 11), none of these is close enough to root a *B. anthracis* PA phylogenetic tree. Without an ancestral PA sequence, one is unable to determine which PA phenotypes are ancestral and which are derived.

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